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Guidance receptors find their places in the axonal order

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Abstract

In this issue, Katsuki and colleagues show that cell-autonomous mechanisms divide *Drosophila* axons into proximal and distal compartments. Axon guidance receptors selectively localize to one compartment. A diffusion barrier exists near the compartment boundary, suggesting that it may have properties like those of the axon initial segment in mammalian neurons.

Axonal cell surface proteins in the embryonic insect CNS sometimes exhibit striking localization patterns which suggest that they are segregated into specific regions of developing axons. One of the first examples of this was the observation that grasshopper Fasciclin I and II are localized to commissural and longitudinal axon tracts, respectively(Bastiani et al., 1987) (Figure 1). Most interneurons in the embryonic CNS extend axons across the midline in one of the two commissural tracts, and then turn anteriorly or posteriorly along the longitudinal tracts. Thus, restriction of a protein to commissural or longitudinal tracts suggests that it is selectively localized to the proximal or distal portions of axons. However, the mechanisms by which this localization is accomplished are largely unknown.

A paper in this issue of *Neuron* (Katsuki et al., 2009) describes experiments to address whether restriction of membrane proteins to proximal or distal axonal segments can be determined within an individual *Drosophila* neuron, or is a property of the neuron only in the context of the other cells in the developing CNS. The authors studied isolated neurons in primary cell cultures from embryos. They found that two axon guidance receptors, Roundabout2 (ROBO2) and ROBO3, are localized to the distal segment of the axon, while another receptor, Derailed (DRL), is localized to the proximal segment. ROBO2 and ROBO3 are localized to longitudinal axons *in vivo*, while DRL is on one of the commissures (see(Bonkowsky et al., 1999; Simpson et al., 2000), and references therein). Thus, these data suggest that localization of these three proteins *in vivo* could be partially defined by a cell-autonomous mechanism. However, ROBO, which is also limited to longitudinal axon bundles *in vivo*, has a uniform distribution along the entire length of the axon in cultured neurons.

If the same axonal structures are used for localizing proteins to the proximal and distal axon segments, the authors reasoned that proximal and distal proteins should respect a common boundary. This was in fact observed. The proximal boundary of ROBO3 expression corresponds to the distal boundary of DRL expression. When ROBO2 and ROBO3 were labeled at the same time, the proximal boundary of expression was common to both

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receptors, suggesting that the distal segment represents a unit that is not subdivided, at least for these two receptors.

Since membrane addition is likely to occur at the distal end of the growing axon, these distinct patterns of localization could be set up by temporally ordered expression. A temporal control model predicts that proximal membrane proteins should be expressed at an earlier time point than distal proteins, and that the timing of expression is critical in determining localization within the axon. To test this hypothesis, Katsuki *et al*. constitutively expressed the receptors, and found that they still localized normally to compartments. They also showed that if a pulse of expression of ROBO3 is induced after axons have begun to extend, ROBO3 is loaded into both the new segment of the axon and into the portion of the old segment that is distal to the compartment boundary. In summary, these data suggest that the axons are intrinsically divided into two compartments, and that protein localization to proximal or distal segments is due to directed trafficking into the appropriate compartment, or to selective retrieval from the inappropriate compartment

To examine if endocytosis and retrieval are involved in compartmentalization, Katsuki et al. used a temperature-sensitive allele of *shibire (shi)*, the fly dynamin ortholog. Dynamin is required for clathrin-dependent endocytosis, and shi^{ts1} mutants exhibit endocytosis defects at the nonpermissive temperature. shi^{ts1} neuronal cultures were allowed to extend axons at the permissive temperature, and the cultures were later shifted to the non-permissive temperature. Expression of receptors was switched on at the time of the shift, so that they could examine targeting of newly synthesized receptors under conditions in which endocytosis was reduced or eliminated. Loss of dynamin function had a strong effect on the proximal localization of DRL, causing it to become uniformly distributed. When the cultures were shifted back to the permissive temperature, DRL localization was restored. Thus, dynamin-dependent endocytic mechanisms may contribute to localization of DRL to the proximal compartment. However, DRL may still be trafficked to the proximal compartment after synthesis, because when it was examined shortly after the shift to non-permissive temperature it exhibited proximal localization. Perhaps DRL is initially targeted to the proximal compartment but can then leak into the distal compartment. Distally localized DRL might be removed by distal compartment-specific endocytosis. Dynamin function is not required for distal compartmentalization of ROBO3.

One mechanism for compartmentalizing axons into proximal and distal segments would be to employ a barrier to diffusion of membrane proteins. In order to determine if cultured *Drosophila* neurons have a diffusion barrier at the boundary between the proximal and distal compartments, Katsuki *et al*. conducted fluorescence recovery after photobleaching (FRAP) experiments. When membrane targeted mCD8-GFP (a transmembrane protein), which is uniformly distributed along the axon, is bleached at various positions, recovery of GFP fluorescence from across the compartment boundary is much slower than at positions distant from the boundary. These data suggest that movement of CD8-GFP through the boundary region is impeded. The same result is found with glycosyl-phosphatidylinositol-linked-GFP, which is found in the outer leaflet of the membrane, but not with GAP-GFP, which is localized to the inner leaflet. Using spot-size FRAP experiments, in which only a small segment of the axon is bleached, the authors were able to show that the boundary region exhibits reduced recovery rates, with a larger fraction of the tagged protein being immobile. This effect is restricted to a region about 10 μm in length that spans the boundary between the proximal and distal compartments.

Finally, Katsuki *et al*. asked whether proteins needed for presynaptic terminal development use the same compartmentalization mechanisms. They observed that the synaptic vesicle proteins Synaptotagmin and Synaptobrevin localized to the distal segments of the axon, with

a proximal boundary corresponding to that of ROBO3. Thus, new synaptic proteins might be initially directed into the correct region of the axon using the same mechanisms employed for distal localization of guidance receptors.

In summary, Katsuki *et al*. have shown that isolated *Drosophila* neurons localize transmembrane receptors and synaptic vesicle proteins into proximal and distal compartments. The extent to which this compartmentalization reflects the *in vivo* localization of these proteins remains to be determined. ROBO, which is restricted to longitudinal tracts in late embryos, is uniformly distributed along cultured axons. Also, the two surface molecules exhibiting proximal localization, DRL and the antigen recognized by monoclonal antibody (mAb) BP102 (Seeger et al., 1993) have very different distributions *in vivo*. DRL is expressed on the commissural segments of a subset of CNS axons, while BP102 antigen is expressed on both commissural and longitudinal tracts, and is likely to be uniformly distributed along all interneuronal axons. BP102 antigen can be considered to be proximal, however, in motor neurons, because it stains only those portions of motor axons that are within the boundaries of the CNS. Motor axon segments in the periphery do not stain. This also raises the question of what types of neurons are being examined here, and whether they can all be considered to have the same properties. Neuronal cultures derive from early embryos that contain only neuroblasts. These neuroblasts settle on the coverslips and generate neuronal lineages, which include intersegmental interneurons, local interneurons, and motor neurons. These neuronal types express different markers and might have different compartmentalization properties.

Although this is the first study of axonal compartmentalization in cultured *Drosophila* neurons, the concept of a diffusion barrier in the proximal axon segment is not new. Mammalian neurons have a compartment known as the axon initial segment (AIS), which contains specific cytoskeletal and cell adhesion proteins. In cultured hippocampal neurons, the AIS functions as a diffusion barrier that limits the exchange of membrane proteins between the somatodendritic and axonal compartments, as well as between the proximal and distal segments of the axon (Winckler et al., 1999). The diffusion barrier in the AIS is eliminated by agents that disrupt the actin cytoskeleton or the membrane. The AIS also restricts diffusion of phospholipids(Nakada et al., 2003).

In the cultured *Drosophila* neurons used in this study, the diffusion barrier around the proximal-distal boundary is farther from the soma than is characteristic of the AIS in cultured hippocampal neurons. However, since the dendrites of insect neurons do not connect to the soma, but rather to the proximal segment of the axon, a boundary that prevents protein movement between the dendrites and axon (as the AIS does in mammals) would have to be distal to the point at which the dendritic tree joins the axon. Recent data show that cytoskeletal proteins can exhibit restriction to proximal axon segments in *Drosophila* brain neurons *in vivo*, suggesting that these neurons may have an AIS-like region. In these cases, the region demarcated by cytoskeletal protein localization extends distally beyond the attachment point of the dendrites (Rolls et al., 2007).

A major organizer of the mammalian AIS is thought to be the cytoskeletal scaffolding protein ankyrinG, which interacts directly with transmembrane cell adhesion molecules and ion channels. When ankyrinG protein expression is knocked down with RNAi, neurons lose polarity and axons begin to express dendritic markers(Hedstrom et al., 2008). These data suggest that loss of ankryinG eliminates the diffusion barrier in the AIS.

It will be of interest for the future to determine whether the diffusion barrier in *Drosophila* axons also requires ankyrin function, and if loss of ankyrin can cause axonal proteins to localize to the wrong compartments. There are two ankyrin genes in *Drosophila, Ank* and

Ank2. Ank protein is ubiquitously expressed, while Ank2 is found primarily in the developing nervous system. A large isoform of Ank2 is selectively localized to axons, although not to specific axonal segments(Hortsch et al., 2001). Ank2 is required for synaptic stability at the larval neuromuscular junction (Koch et al., 2008; Pielage et al., 2008).

The system described in the Katsuki *et al*. paper opens the door to a genetic analysis of the formation of axonal compartments and diffusion barriers. Neuronal cultures can be made from any *Drosophila* mutant strain, and expression of fluorescently tagged transmembrane proteins can be induced in a temporally and spatially controlled manner. Cultures can also be made from embryos expressing transgenic RNAi constructs in all neurons or in specific neuronal subsets. Such analyses could define the cytoskeletal or membrane proteins that are required for compartment formation and maintenance in all classes of neurons.

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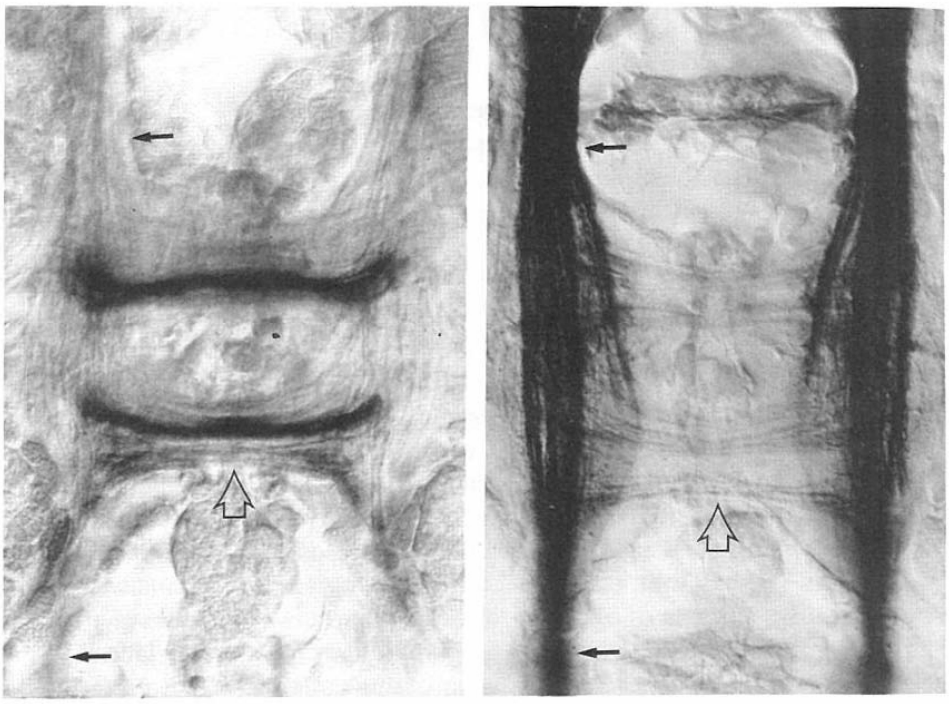

Figure 1. Restriction of surface proteins to specific axonal regions *in vivo*

Fasciclin I (left panel) is restricted to one bundle in each of the commissures (empty arrows) of the T2 segment of the embryonic grasshopper CNS. Fasciclin II (right panel) is localized to longitudinal fascicles (black arrows). Modified from Bastiani, M.J. *et al.* (1987). Cell 48, 745-755.